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Collagen proteins exchange O with demineralisation and gelatinisation reagents and also with atmospheric moisture

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Short title: Oxygen isotope analysis of mammalian skeletal collagen

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Abstract

RATIONALE: The oxygen isotope composition of collagen proteins is a potential indicator of adult residential location, useful for provenancing in ecology, archaeology and forensics. In acidic solution, proteins can exchange O from carboxylic acid moieties with reagent O. This study investigated whether this exchange occurs during demineralisation and gelatinisation preparation of bone/ivory collagen.

METHODS: EDTA and HCl demineralisation or gelatinisation reagents were made up in waters with different $\delta^{18}O$ values, and were used to extract collagen from four skeletal tissue samples. Aliquots of extracted collagen were exposed to two different atmospheric waters, at 120°C and ambient temperature, and subsequently dried in a vacuum oven at 40°C or by freeze drying. Sample $\delta^{18}O$ values were measured by HT/EA pyrolysis-IRMS using a zero-blank autosampler.

RESULTS: Collagen samples exchanged O with both reagent waters and atmospheric water, which altered sample $\delta^{18}O$ values. Exchange with reagent waters occurred in all extraction methods, but was greater at lower pH. Damage to the collagen samples during extraction increased O exchange. The nature of exchange of O with atmospheric water depended on the temperature of exposure: kinetic fractionation of O was identified at 120°C but not at ambient temperature. Exchange was difficult to quantify due to high variability of $\delta^{18}O$ value between experimental replicates.

CONCLUSION: Studies of $\delta^{18}O$ values in collagen proteins should avoid extraction methods using acid solutions.

Keywords

Collagen, protein, oxygen isotope, exchange, extraction method

1 Introduction

Understanding the mobility of organisms and materials across the earth's surface is of great importance to fields as diverse as ecology, agriculture, sociology, forensics and archaeology.[1-5] The incorporation of local precipitation O and H into body tissues via diet and drinking water leads to robust relationships between precipitation and tissue $\delta^{18}O$ and $\delta^{2}H$ isotope values, which have been successfully exploited to provenance animal and vegetable tissue at continental and regional scales. In ecology, where non-invasive sampling is paramount, work has focused on the $\delta^{2}H$ values of protein in keratinous tissues.[6-11] In archaeology, where the most commonly recovered organic tissues are skeletal, such provenancing work on both humans and animals has focused on biomineral $\delta^{18}O$ values in tooth enamel, either in carbonate or in phosphate.[3, 12-14] Measurement of carbonate and phosphate $\delta^{18}O$ values in the biomineral fraction of bone, which is renewed throughout life,[15] is not usually suitable as these values have been shown to be affected by post-depositional diagenesis[16-20] although they have been useful in forensic applications.[21] The isotopic composition of human tooth tissues, however, reflects only dietary ingesta and drinking water imbibed during infant, juvenile and sub-adult years, when teeth are formed.[22] It would be useful to have a robust analytical indicator of dietary and drinking water inputs in the longer period of adult life. The $\delta^{18}O$ values recorded in the protein fraction of bone provide such an alternative data source. This has so far been little used for provenance information, although its applications are becoming more commonly reported.[23-27]

Isotopic analysis of the protein fraction of bone, which is largely composed of collagen, first requires that the biomineral from the sample be removed. In most laboratories and applications,[28, 29] this is carried out using a version of the Longin method, a 2-stage demineralisation and gelatinisation protocol, both involving mineral acid, HCl(aq). Alternative methods have been developed, using aqueous solutions of either an organic acid, acetic acid,[30] or the ion chelating agent, ethylenediaminetetraacetic acid (EDTA).[31, 32] All these demineralisation methods were originally developed to extract collagen for radiocarbon dating or for palaeodietary studies focusing on carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values) isotope analysis of the extracted protein. For analyses targeting O in proteins, methods employing acid solutions are problematic, as the O atoms in amino acid carboxyl groups exchange with O in solvent water at low pH.[33] This phenomenon is the basis of a quantitative labelling technique in proteomics.[34] It is therefore likely that collagen extraction

methods employing acid solutions will introduce O from reagents into the protein, thus altering the endogenous $\delta^{18}O$ value obtained from the collagen.

The magnitude of this effect is potentially large. O exchange sites include the terminal carboxyl group of the total peptide, the side chain O of all glutamic acid (Glu) and aspartic acid (Asp) residues,[33] and the peptide O in Asp and asparagine (Asn) residues, due to within-chain racemisation via the formation and subsequent hydrolysis of the succinimidyl residue.[35] The rate of this reaction is faster if the collagen is gelatinised.[36] In theory, the peptide O of Glu may also exchange via a cyclic intermediate in aqueous solution.[37] The recently reported within-chain racemisation of serine (Ser)[38, 39] will conversely not increase O exchange further. In collagen, Glu, Asp and Asn make up 9% of all residues: 13-14% of the O in the protein is therefore exchangeable (collagen sequences from http://www.uniprot.org/). Longin-type laboratory preparation of collagen for isotopic analysis could therefore markedly reduce the accuracy of provenance assignment based on this data by accelerating carboxyl side-chain O exchange and increasing the extent of succinimide formation. This would prohibit comparison of collagen $\delta^{18}O$ data between protocols and laboratories. This effect is likely to be stronger in bone samples that are poorly preserved or structurally altered due to diagenetic alteration,[32, 40-42] which may introduce significant bias between samples obtained from different sites or from different contexts within a site.

In addition, in extracted collagen, it is possible that O exchange between the protein and atmospheric moisture also occurs, during storage or analysis. Residual acid from the gelatinisation step in the Longin method, which is not removed, may continue to catalyse O exchange in extracted collagen in the presence of adsorbed atmospheric water. This process is analogous to the well-known process of protein H exchange with atmospheric water,[43, 44] but in this case requiring a catalyst. The duration and nature of collagen storage and the isotopic composition of local atmospheric water may therefore also contribute to measured collagen $\delta^{18}O$ values.

However, the rates of these reactions appear to be relatively slow. Acid-catalysed O exchange is much slower than trypsin-mediated O exchange.[11, 45] Exchange at the C-terminus is faster than exchange at carboxyl acid side chains.[34] The extent of exchange therefore is likely to depend not only on the pH of the reagent but also on the period of exposure. Apparent exchange values could also be affected by incomplete drying of the sample before measurement.[43]

This study investigated the degree of acid-catalysed O exchange during standard laboratory preparation of bone collagen, by comparing a non-acid extraction method (EDTA) with a Longin-type (HCl-based) protocol. The effect of HCl demineralisation only (first step of the Longin method)[46] was compared with that of the total 2-step process, including gelatinisation. The study also investigated the potential for acid-catalysed O exchange mediated by the presence of atmospheric moisture and residual HCl from preparation solutions. The equilibration conditions were adapted from experiments on keratin.[43] Protein integrity was monitored via carbon and nitrogen elemental and isotopic composition,[47, 48] amino acid composition and degree of racemisation.[49, 50]

This study tested the following hypotheses:

- Incorporation of O from aqueous reagents into bone collagen significantly affects collagen $\delta^{18}O$ values.
- Incorporation of O from atmospheric moisture into demineralised bone collagen does not significantly affect collagen $\delta^{18}O$ values.

In both cases:

- Exchange of O is larger in samples prepared by demineralisation with HCl than by demineralisation with EDTA.
- Exchange of O is larger in samples demineralised and gelatinised using HCl than in those only demineralised by HCl.
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2 Experimental

2.1 Sample origin

Four samples of mammalian tissue were obtained from widely differing climatic regimes, which were expected to generate widely differing $\delta^{18}O$ values.[51-53] These were: African elephant (*Loxodonta africana* or *cyclotis*) ivory from the Democratic Republic of the Congo (then Zaire, seized in the 1980s under CITES legislation[54]); a modern domesticated cattle (*Bos taurus*) femur from the UK (supplied by Oliver E. Craig, University of York); a modern semi-domesticated reindeer (*Rangifer tarandus*) humerus from Finland (supplied by Auli Bläuer, University of Turku); and a fragment of mammoth (*Mammuthus* sp.) long bone of indeterminate age from Yukon, Canada, supplied by Grant Zazula, Government of Yukon Department of Tourism and Culture. Samples were cut or crushed into small cubes or chips,

approximately 1-3 mm per side. Duplicate sub-samples of 0.5 g were used for all experiments.

2.2 Reagent and equilibration waters

For each collagen extraction method, three parallel sets of reagents were made up volumetrically using waters of widely differing $\delta^{18}O$ values (Table 1). These were: tapwater from Keila, Estonia (supplied by Tõnu Martma, Tallinn University of Technology, Tallinn, Estonia; water EE); laboratory water from BioArCh, University of York, York, UK, purified by deionisation, reverse osmosis and ultrafiltration (ELGA Purelab Ultra, Marlow, UK; water DIY); and a ^{18}O-enriched water made by evaporating batches of DIY water to 25% volume (water 75E). The Estonian tapwater was used without filtration.

Equilibration waters were: (1) water EE and (2) a second ^{18}O-enriched water made by evaporating batches of laboratory water from the Archaeological Stable Isotope Laboratory, University of Kiel (Kiel, Germany), purified by deionisation, reverse osmosis and deionisation (RiOs-DI 3UV, Millipore, Billerica, MA, USA), to 25% volume (water K75E).

2.3 Collagen extraction

Stock solutions/reagents for all reagents were as follows: EDTA powder (MP Biomedicals, Illkirch, Germany), 37% HCl (aq) (analytical reagent grade, Fisher Scientific UK, Loughborough, UK), and NaOH pellets (analytical reagent grade, Fisher Scientific). Extractions were performed in borosilicate glass sample vials (Fisherbrand, Fisher Scientific UK).

2.3.1 Extraction method 1: EDTA (aq)

Bone samples were placed in 0.5 M EDTA (aq, pH 7.5; pH was adjusted by addition of 2 M HCl (aq)) with the solution replaced every 2-7 days until demineralisation was complete,[55], for a minimum of 14 days. The collagen pseudomorph was rinsed in ultrapure water 15 times to remove residual EDTA solution, with a rinse in 0.1 M NaOH (aq) after the seventh water rinse in order to remove humic acids, followed by an overnight soak in water on the eighth rinse to remove residual EDTA solution.

2.3.2 Extraction method 2: HCl demineralisation only

The bone sample was demineralised in 0.6 M HCl (aq) at 4°C for a minimum of six days. The collagen pseudomorph was rinsed in water six times, with an overnight soak at 4°C after the third rinse, to neutrality.

2.3.3 Extraction method 3: HCl demineralisation and gelatinisation

The bone sample was demineralised as in extraction method 2, but rinsed only three times in water. The sample was gelatinised in pH3 HCl (aq) at 80°C for 48 hours. The supernatant containing gelatinised collagen was filtered (Ezee Filter 9mL separators, 60-90µm pore, Elkay Laboratory Products, Basingstoke, UK), and ultra-filtered (30 kDa, Amicon Ultra-4 centrifugal filter units, Millipore; filters pre-rinsed with 0.1 M NaOH (aq)). The retentate collagen fraction was preferred for isotopic analysis (but see section 3.1).

All samples were frozen then freeze-dried for >24 hours after extraction, and subsequently stored in ambient conditions in the laboratory. The storage time was 3-8 months for most samples, but the EE/EDTA extractions were only stored for c. one week.

2.4 Equilibration

Aliquots of prepared collagen (0.5 mg) were weighed into 4 × 3.2 mm silver capsules (Elemental Microanalysis, Okehampton, UK). A single suite of 72 samples (one each for every combination of sample, reagent water, collagen extraction method, and duplicate) was used for each equilibration experiment. Equilibration conditions were chosen to explore the range of possible exchangeabilities, by analogy to previous work with keratin samples.[43]

2.4.1 Equilibration method 1: high temperature/oven drying

Capsules for high-temperature equilibration were placed into 1.5-mL glass vials (Supelco, Sigma-Aldrich, St. Louis, MO, USA) in a steel frame. The loaded frame was placed in a vacuum oven (Thermo Fisher Scientific, Waltham, MA, USA) at ambient temperature, with a beaker containing 10 mL of the frozen equilibration water. The oven was evacuated with a vacuum pump (Adixen Drytel, Pfeiffer Vacuum Technology, Aßlar, Germany) to c. 200 mb pressure to remove air but the minimum of equilibration water, and heated to 120°C. After 2 hours, the oven temperature was reduced to 40°C overnight. The samples were then dried under vacuum (<0.1 mb) at 40°C for 4 days.

2.4.2 Equilibration method 2: ambient temperature/oven drying

Samples for ambient equilibration were placed in a 96-microwell plate (Nunc, Thermo Scientific). The plate was placed in a glass desiccator with a beaker containing 10 mL of the frozen equilibration water. The desiccator was placed under vacuum using a diaphragm pump (Vacuubrand, Wertheim, Germany) for 7-8 days at ambient laboratory temperature, c. 20°C. The samples were then dried in a vacuum oven (as above, <0.1 mb) at 40°C for 4 days.

2.4.3 Equilibration method 3: ambient temperature/freeze drying

Samples were equilibrated at ambient temperature as in section 2.4.2 but were subsequently dried in a freeze drier (Lablyo, Frozen in Time Ltd, Sheriff Hutton, UK) under vacuum (<0.1 mb) for 3 days (EE water sample set) or 4 days (K75E water sample set).

2.5 Determination of isotopic composition

Reagent and equilibration water $\delta^{18}O$ values were determined by isotope ratio infrared spectroscopy (IRIS) based on wavelength-scanned cavity ring-down spectroscopy (L 1102-i WS-CRDS, Picarro Inc., Santa Clara, CA, USA) at the Hydrogeology Stable Isotope Laboratory, FAU Erlangen-Nürnberg, Germany.[56] Four sequential injections of each sample were measured and raw data were corrected for sample-to-sample memory. The reported value is the mean value. The data sets were corrected for instrumental drift during the run and normalized to the VSMOW scale using two internal laboratory reference materials. All values are reported in the standard δ-notation in per mil (‰) vs Vienna Standard Mean Ocean Water (VSMOW). The external reproducibility based on repeated analyses of a control sample was better than 0.1‰ (±1 σ).

Collagen $\delta^{18}O$ values were determined using an HT-EA pyrolysis unit (glassy carbon reactor) linked to a 20-20 isotope ratio mass spectrometer in continuous flow mode, with a zero-blank autosampler (all manufactured by Sercon, Crewe, UK). For transfer to the mass spectrometer, collagen samples (already in Ag capsules) were removed from the oven/freeze drier, parafilmed in their containers and transported between laboratories in a plastic bag containing silica bead desiccant. The total time from vacuum break to start of autosampler purge was always <10 minutes. Only 30 unknowns could be loaded into the autosampler at once, so a single suite of 72 samples comprised 3 separate analytical runs, with unused samples being replaced in the oven/freeze dryer under original drying conditions until used. The total time from vacuum break to replacement of the samples under original conditions was always <5 minutes; the total time to run any single suite of samples was always <36 hours. Each suite of samples was run in the same order, with all duplicate 1 samples being followed by all duplicate 2 samples. All values are reported in ‰ notation vs VSMOW. The protein standards were chitins (FM203, Forensic Isotope Ratio Mass Spectrometry (FIRMS) Proficiency Testing (PT) scheme, LGC Standards, Teddington, UK; and Chitin Standard Certificate 121220, Elemental Microanalysis). Non-protein standards were nylon (FM221, FIRMS PT scheme), and benzoic acids (IAEA 601 and IAEA 602, International Atomic Energy Agency, Vienna, Austria). The separation between the N_2 and CO peaks was not

total, so the data were corrected offline by manual selection of an NO-free baseline value.[57] The maximum within run-uncertainty (1σ, n=2-3) for the chitins and IAEA 601 was 0.5‰; for the nylon it was 0.4‰ (median 0.2‰); and for IAEA 602 it was 2.6‰ (median 1.1‰). The standard accuracy and precision data are given in Table SI1 (supporting information) .

Collagen $\delta^{13}C$ and $\delta^{15}N$ values were determined using a GSL preparation unit linked to a 20-20 isotope ratio mass spectrometer in continuous flow mode (manufacturer as above). Collagen samples were weighed into 4 × 3.2 mm tin capsules (Elemental Microanalysis) without atmospheric equilibration. Standards were freshwater fish gelatine (Sigma-Aldrich), cane sugar (R006, Iso-Analytical, Crewe, UK), caffeine (IAEA 600) and ammonium sulfate (IAEA N2). Maximum within-run uncertainty (1σ, n=3-13) was 0.04‰ for $\delta^{13}C$ and 0.2‰ for $\delta^{15}N$.

2.6 Amino acid composition and degree of racemisation

To examine the effect of workup methods on protein integrity, amino acid composition and racemisation ratios were determined for all cattle sample aliquots (n=18) by reversed-phase high-performance liquid chromatography (RP-HPLC) following the methodology for bone samples in Buckley et al,[58] except that samples had previously been demineralised and/or gelatinised before hydrolysis with 7M HCl (aq) under N_2 at 110 °C for 18 h. With this RP-HPLC method, the concentration of L- and D-enantiomers of nine amino acids is measured; a further three amino acids are recovered as L-enantiomers only; Gly is also recovered but does not have stereoisomers. The data are reported as the concentration of each amino acid (indicated [AA]), the percentage of recovered amino acids (indicated [AA]%) and the racemisation ratio (D/L). Asparagine (Asn) and glutamine (Gln) are deamidated during preparative hydrolysis to aspartic acid (Asp) and glutamic acid (Glu), respectively, so Asp and Asn are reported together as Asx, and Glu and Gln as Glx.

The measurement error per sample (n=2, 1σ) was 0-1% for AA%s, with the exception of Gly (4%), and 0.00-0.02 for DL ratios, with the exception of Arg D/L and Leu D/L (0.08).

2.7 Statistical treatment

All statistical analysis was carried out using R.[59]

Equilibration calculations assumed that all O in collagen protein is either acid-exchangeable or completely non-exchangeable, and that there was no isotopic fractionation between the exchangeable O and water vapour, by analogy with protein H exchange with atmospheric

moisture.[43] The mole fraction of exchangeable O (X_o) in a sample of collagen exposed to two waters (A and B) with different $\delta^{18}O$ values was therefore:

$$X_O = \frac{\delta^{18}O_{coll,A} - \delta^{18}O_{coll,B}}{\delta^{18}O_A - \delta^{18}O_B}$$

3 Results and discussion

The full $\delta^{18}O$ data are presented inSI2(supporting information), the $\delta^{13}C$ and $\delta^{15}N$ data in SI3 (supporting information , and the AA data in SI4 (supporting information). The data were non-parametric (Shapiro-Wilk test, P<<0.001) and no effective data transformations were found.

3.1 Collagen quality

The collagen preparation yielded retentate material of good quality and quantity for 68 of the 72 samples. The total collagen mass yields (filtrate + retentate) were 17-24% for bone samples and 25-33% for ivory samples. All these samples had acceptable $C:N_{atom}$ ratios,[47, 48] with mammoth, cattle and elephant samples showing a range of 3.10-3.25 and reindeer samples a range of 3.20-3.45. The mass C and N contents were 42-49% and 15-18%, respectively. These are higher than the values expected for archaeological material[47] and are closer to the theoretical elemental mass percentages calculated for intact collagen sequences (51% and 17%, respectively).

In cattle samples, the degree of amino acid racemisation was very similar between EDTA and HCl-demineralised samples, being measurable in Asx (0.05), Glx, Arg and Ala (0.02-3), and zero for all other AAs. Samples prepared by HCl demineralisation and gelatinisation showed higher racemisation ratios than EDTA or HCl-demineralised samples, especially in Asx (0.1-0.12), but also slight elevations for Glx, Arg and Ala D/Ls. There were no differences between samples extracted in different waters. This indicated that HCl gelatinisation increased racemisation of amino acids in extracted collagen. This effect is most significant for Asx, where the extent of racemisation increased from 0.05 (5% of Asx residues) to 0.11 (10% of Asx residues). Between 0.6 and 1.1% of total protein O (depending on the degree of deamidation of Asn to Asp) could therefore potentially exchange with reagent water during racemisation induced by HCl gelatinisation. The baseline Asx DL ratio of 0.05[49] indicates some damage to the protein during demineralisation or, probably, during the preparative acid hydrolysis for the AAR analysis itself.[60] This value is therefore the upper limit of the degree

of racemisation expected in the sample aliquots on which isotope analysis was carried out, equivalent to the exchange of 0.3-0.5% of total protein O.

Four samples were compromised during laboratory preparation: the cattle and elephant aliquots prepared using HCl demineralisation and gelatinisation in DIY water. These yielded no or little filtrate and a sticky pale yellow oil as retentate, instead of the off-white flaky foam typical of this preparation method. A pH check on the reagents indicated that the nominally pH3 HCl solution in DIY water erroneously had a pH of 2. This was corrected before the reagent was used for reindeer samples, and but had not apparently affected the mammoth samples. No usable sample material was available for the elephant samples, but filtrate cattle material (off-white sticky lumps) was investigated for amino acid composition, and carbon and nitrogen elemental and isotopic composition. These samples showed abnormally low %C and %N content. They had lower protein contents per gram (40-50% reduced) than analogous samples extracted with EE and 75E reagents. They showed clear loss of Ser, L-Thr, L-Arg and (relative) gain of Glx, Ala and Leu. They had unprecedentedly high racemisation ratios for bone samples for all amino acids measured (1.3-0.3).[49, 50] This indicates that these samples had been partially hydrolysed during gelatinisation. They were probably also fully deamidated.[41] The Asx D/L ratio indicates the racemisation of 46% of Asx residues, equivalent to the potential exchange of 3-5% of total protein O, depending on the degree of deamidation. The compromised cattle samples were investigated for their $\delta^{18}O$ values together with all the intact samples, but the data was excluded from statistical analysis.

3.2 Collagen $\delta^{18}O$ ranges

As expected from the relative predicted precipitation $\delta^{18}O$ values of the samples' approximate locations of origin, the $\delta^{18}O$ values were highest in elephant samples, then in cattle and reindeer, with mammoth samples yielding the lowest values (Table 2). All four sample groups had significantly different $\delta^{18}O$ ranges (Mann-Whitney test with Bonferroni correction, all $P<0.001$). Elephant ivory yielded the widest range (c. 22‰); bone samples yielded smaller ranges (mammoth 10‰; cattle 10‰; reindeer 5‰). These ranges are high compared with the mammalian total range (35‰ in this study) and even the total global precipitation $\delta^{18}O$ range of 45‰ for monthly observations.[61]

The reproducibility of the $\delta^{18}O$ values was not good: differences in $\delta^{18}O$ value between identically treated experimental replicates (n=2 in each case) was 0-2.3‰ for mammoth bone (with one outlier at 4.6‰, median 0.6‰), 0-3.7‰ for reindeer bone (median 0.6‰), 0-2.1‰

for cattle bone (with two outliers at 4.7‰, median 1.0 ‰) and 0-4.9‰ for elephant ivory (with one outlier at 7.8‰, median 1.6‰).

Poor reproducibility was in part due to N_2 interference with the CO peak in the mass spectrometer. The reindeer samples showed the highest reproducibility, probably because they had the lowest N content of all sthe amples (indicated by relatively high $C:N_{atom}$ ratios). This interference was minimised but could not be completely eliminated by baseline correction. However, the fact that collagen samples had greater variability than chitin standards indicates that an additional contributing factor to O variability was present. This could be variable degrees of damage to the collagen molecule during workup, causing different degrees of exchange with ambient water O. Assuming that the high reproducibility of the degree of racemisation measured between duplicate cattle samples is generalisable to the material from other species, then the low reproducibility of the $\delta^{18}O$ values was not due to variability in protein damage caused by protein extraction. Thermal or chemical damage over time may have had an effect, as the mammoth samples had the highest variability of all bone samples analysed. Although these samples showed no macroscopic, elemental or amino acid indication of protein damage, none of these methods are sensitive to deamidation, which does affect O exchangeability. This could have been directly assessed via mass spectrometric techniques.[40, 41, 62, 63] In the presence of $H_2{}^{18}O$ it is possible to monitor the direct incorporation of O at specific residues,[34] which can be mapped using LC/MS/MS and quantified using tools such as iTRAQ.[64] Like racemisation, which is accelerated at the N-terminus, mass spectrometry can identify points of chain scission at sites which would not typically be cleaved during sample preparation, but which might occur during burial over archaeological timescales. For archaeological applications, examination of how much O exchangeability can vary between bone samples of different preservation states is essential.

We consider that endogenous variability in collagen $\delta^{18}O$ values within the sample remains the most likely driver for the observed tissue $\delta^{18}O$ ranges. This is also suggested by: (1) the greater variability recorded in ivory samples than in bone samples, as ivory grows incrementally without reabsorption,[65] whereas bone is continually grown and reabsorbed,[15] which will tend to reduce the amplitude of isotopic variation recorded; and (2) the relative homogeneity of values for samples treated with the full Longin method, which were averaged by dissolution. In this study of herbivorous species, such endogenous variability reflects variability in $\delta^{18}O$ values in dietary and drinking water inputs, which is dependent on their ranges of precipitation inputs, evaporative processes in local surface water bodies, and plant

responses to water availability,[66-68] and which are recorded differently depending on the growth period and pattern of the tissue type sampled.[24, 25, 69] The recorded variability in herbivore tissues can be large, e.g. cattle tail hair records an annual variation of 5-8‰ in temperate Europe, about the same as the local annual precipitation range,[70] while dentine collagen samples from Mongolia record seasonal ranges of 9-11‰ per individual, which is smaller than the local annual precipitation range of c. 18‰.[24] The ranges of variability recorded in this project are therefore not inherently unlikely, especially as collagen remodelling appears to be very slow.[69, 71, 72] In addition, metabolic variation in mammalian body water $\delta^{18}O$ values due to ontogeny and nutritional adequacy is also likely to be a contributing factor.[26, 70, 73, 74] This variation should be characterised before bulk collagen $\delta^{18}O$ values can be robustly used for provenancing.[27]

3.3 Effect of equilibration with atmospheric water

There was no significant difference in $\delta^{18}O$ values between samples equilibrated with EE water and those equilibrated with K75E water (P=0.5), consistent with our hypothesis. The variability of measured $\delta^{18}O$ values was, however, always greater for samples exposed to K75E water than for those exposed to EE water (illustrated for mammoth samples in Figure 1).

There were no apparent systematic differences in the degree of calculated atmospheric exchangeability between equilibration methods (Table 3). However, at least two different equilibration mechanisms occurred. For both ambient temperature equilibration methods, samples exposed to ^{18}O-enriched K75E water (high $\delta^{18}O$ values) showed slightly higher measured $\delta^{18}O$ values than those exposed to ^{18}O-depleted EE water, as expected. The isotopic effect of exposure to atmospheric water was, however, inversed for samples equilibrated at high temperature: samples exposed to ^{18}O-enriched K75E water (high $\delta^{18}O$ values) showed lower measured $\delta^{18}O$ values than those exposed to ^{18}O-depleted EE water (illustrated in Figure 2 for mammoth samples). This suggests the presence of the kinetic isotope effect identified for keratin in water at 140°C,[75] in which heated samples exchanged ^{16}O faster than ^{18}O, leading to ^{18}O-enrichment of the residual protein. In the present study, this effect was probably greater with K75E water than with EE water because of its lower pH, leading paradoxically to lower $\delta^{18}O$ values in samples exposed to water of higher $\delta^{18}O$ value. This fractionation means that the assumptions behind the calculation of X_o from $\delta^{18}O$ values (see section 2.6) did not hold for samples equilibrated at high temperature. X_o values calculated by this method were not valid for this subset of samples.

Sample O exchangeability (X_o) due to ambient equilibration with atmospheric moisture was calculated pairwise by comparison of measured $\delta^{18}O$ values for EE-exposed and K75E-exposed samples for each sample/extraction method/ambient equilibration method combination, according to the equation in section 2.7 (n=12 in each treatment group, Table 3). The values ranged between 0% and 7%. There were no differences in X_o between species for bone samples (1-4%; Table 3, Figure 3). The ivory X_o values were higher (2-8%). The compromised cattle samples showed much higher X_o (20-30%), probably due a combination of the increase in C-termini due to hydrolysis, and the lower energy barrier to succinimide formation in gelatine than in collagen.[36] These samples were probably also fully deamidated, which increases the total theoretical O exchangeability of the protein to 20%. However, the errors (calculated in quadrature) were relatively large (3-66%, median 7%), as duplicate reproducibility of $\delta^{18}O$ values was low.

There were no differences in X_o between extraction methods, indicating that all collagen produced interacted to an equal degree with atmospheric moisture. This indicated that neither the presence of residual acid nor the conformational freedom of the collagen molecules (gelatinised or still in H-bonded trimer helixes)[76] controlled O exchange at either high or ambient temperature.

An additional source of uncertainty in these calculations is fractionation between the liquid and gaseous phases in the equilibration chambers. In both the vacuum oven and the desiccators, a large excess of equilibration water (10mL) was supplied, not all of which evaporated. The water to which the collagen samples were exposed will have been more depleted in ^{18}O than the bulk sample. However, we assume that this fractionation was approximately equal in EE and K75E waters, although not between ambient and high-temperature equilibrations.

3.4 Effect of extraction method and reagent water

Sample O exchangeability (X_o) with reagent water was calculated by comparison of the measured $\delta^{18}O$ values for EE-extracted, DIY- extracted and 75E- extracted samples for each sample/extraction method combination, according to the equation in section 2.7, that is by three pairwise comparisons between the three reagent waters (Figure 4), but are also estimated graphically by linear regression in Figure 5. Only one pairwise comparison was available for cattle and elephant samples. Data from high temperature-equilibrated samples was excluded due to subsequent fractionation (so n=8 in each treatment group). For bone

samples, the O exchangeability with reagent water was 1-7% (median 2%) for EDTA methods, 2-9% (median 7%) for HCl demineralisation only, and 6-11% (median 10%) for HCl demineralisation and gelatinisation. Exchangeability for ivory samples was generally higher and showed the greatest variability with HCl demineralisation (range 4-18%). However, the errors in these values (calculated in quadrature) were large (0-80%, median 8%) and highly variable even for the same sample and extraction method, so should be considered indicative only.

The calculated values for exchangeability with reagent water are within the range for intact collagen proteins (<12%) and in line with the minimal Asx DL ratios measured in cattle samples. However, the general increase in exchangeability for HCl-treated samples suggests some damage to the protein (probably deamidation) during the HCl demineralisation step, which was reflected in the amino acid analysis. The HCl-gelatinised samples showed an increase in exchangeability greater than that which would be consistent with the levels of racemisation observed (0.6-1%), further suggesting deamidation of these samples. The O exchangeability of the compromised cattle samples (12-32%, data not shown) indicated advanced deamidation and racemisation, probably also representing peptide scission. The non-zero O exchange with EDTA(aq) extraction indicates that we erroneously conceived of O in proteins as dividing into “acid-exchangeable” and “non-exchangeable” fractions. Exchange in EDTA solutions is unlikely to be a direct effect of the chelating agent acting on the protein, but can instead be attributed to the solvent water, which even at pH 7-8, is a source of protons that can catalyse O exchange. O in proteins should probably instead be conceived of as divided into “water-exchangeable” and “non-exchangeable” fractions.

The $\delta^{18}O$ values for samples extracted with EDTA showed no clear trend with the reagent water $\delta^{18}O$ values (Figure 5A), in line with the findings of Reynard et al.[77] There were no significant differences in the measured $\delta^{18}O$ values for any EDTA-extracted sample between any pair of reagent waters (all $P>0.05$, after Bonferroni correction; ambient-equilibrated samples only; Figure 5a). Samples extracted with HCl showed clear dependence of the sample $\delta^{18}O$ values on the reagent $\delta^{18}O$ value, with similar behaviour in gelatinised and non-gelatinised samples (Figures 5B and C). For HCl demineralised samples, the $\delta^{18}O$ values were significantly different between EE and 75E waters for mammoth and reindeer samples only ($P<0.05$; Figure 5B). For samples treated with the full Longin method, the $\delta^{18}O$ values were significantly different between all waters for all samples, except for the reindeer and mammoth samples for the DIY-75E comparison ($P<0.05$; Figure 5C).

From this data, it appears that the rate of O-exchange during collagen extraction is faster than suggested previously.[11, 34] The bulk of the exchangeable O in collagen is in the AA side chains, which exchange more slowly under acid conditions than the C-termini, taking >2 weeks to reach equilibrium in ambient temperature and at low pH .[34] In the present experiment, EDTA-extracted samples showed the smallest degrees of exchange, despite having exposure times to reagents measured in weeks. All other reaction steps and equilibration phases were of shorter duration. It therefore remains possible that protein O-exchange with reagents was not complete when samples had finished demineralising, implying that full equilibration with reagents could cause greater exchange than that calculated here.

An additional source of uncertainty in these calculations is the use of the $\delta^{18}O$ value of the solvent water as a proxy for total reagent $\delta^{18}O$ value, which could not be directly measured. This missed potential contributions to reagent O from reagents (EDTA, NaOH, HCl stock solution). The two greatest such contributions are in (1) the 0.5 M EDTA solution, where the solute accounts for up to 7% of the total reagent O atoms in the solution, exchanging with solvent O via the same mechanism as collagen protein AA side chains; and (2) the 0.6M HCl reagent, which contained 3% exchangeable O atoms from the original HCl stock solution. The isotopic composition of the solid reagents will have altered that of the total solution from the original solvent $\delta^{18}O$ value. The magnitude of this effect will depend on the difference in $\delta^{18}O$ values between the solid reagent and the solvent.

4. Conclusion

This study showed that extracted bone/ivory collagen exchanges O with both laboratory reagent water and atmospheric moisture, significantly affecting the sample $\delta^{18}O$ values. Exchange with reagent waters occurred when using all the collagen extraction methods. The use of mineral acid reagents increased the exchange of O compared with a chelating agent, as hypothesised, as did gelatinisation in addition to demineralisation. Exchange of O with atmospheric water in the laboratory did not significantly alter measured $\delta^{18}O$ values, but differed in mechanism between equilibration methodologies. The sizes of these effects were difficult to assess accurately, however, due to high variability in $\delta^{18}O$ value between experimental replicates.

These results indicate that an entirely endogenous $\delta^{18}O$ value from collagen proteins is probably not obtainable, as all laboratory methods used to remove the mineral involve the use of an aqueous solution. Water is itself a proton donor sufficient to catalyse O exchange between solution and carboxylic acid side chains in protein amino acid residues. However, if exchange between protein O and aqueous solution and atmospheric moisture is not avoidable, it can be minimised and standardised. To minimise exchange of O between collagen and reagent solution, we recommend the use of EDTA demineralisation. Collagen extracted using this methodology preserves endogenous spatial variation in $\delta^{18}O$ composition within the original tissue, which is not homogenised via dissolution, and which can be related to changes in the $\delta^{18}O$ composition of environmental inputs during the growth of dentine or bone.[23, 24] The amplitude of variation recorded will nevertheless be blunted by O exchange between protein and reagents, but this effect is likely to be negligible with EDTA extraction. Unfortunately, it is unlikely that the many archived collagen samples generated for radiocarbon dating or palaeodietary studies, and extracted using HCl reagents, are suitable for analysis for $\delta^{18}O$ values, as these samples will all contain a greater contribution from local reagent water at the time of analysis. We recommend that any international standards developed for collagen $\delta^{18}O$ determination should not be demineralised before distribution, but merely be homogenised in powder form. Thus the contributions of local reagent and atmospheric $\delta^{18}O$ values can be assessed for each laboratory.

The susceptibility of proteins to O exchange during aqueous preparation steps and in the presence of atmospheric moisture is common to all proteins, but appears to have been so far unsuspected in isotopic provenancing based on keratinous tissues, although their potential for exchange of H with atmospheric moisture has been well studied.[43, 78, 79] Compared with collagen, these tissues have minimal requirements for sample extraction, and may therefore be less affected by this process. This study highlights that in addition to their metabolic differences, proteins are not necessarily chemically equivalent substrates for provenancing studies.

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Tables

Table 1. Reagent waters used in this study. Errors in $\delta^{18}O$ measurements were <0.1‰ (±1 σ) (see section 2.5).

Abbreviation	Nature	Origin	Use	$\delta^{18}O$ values/‰	pH
EE	Tapwater	Keila, Estonia	Reagent + equilibration	-21.4	7.8
DIY	Ultrapure laboratory water	York, UK	Reagent	-7.6	6.4
75E	Ultrapure laboratory water	York, UK; evaporated	Reagent	10.0	5.9
K75E	Ultrapure laboratory water	Kiel, Germany; evaporated	Equilibration	17.3	6.2

Table 2. Summary of $\delta^{18}O$ isotope compositions data per sample/extraction method /reagent water combination, excluding high-temperature equilibrated samples (see section 3.3). * indicates compromised samples.

				$\delta^{18}O$ value/‰				
Sample	Extraction method	Reagent water	n	min	median	mean	max	range
Cattle	EDTA demineralisation	EE	8	10.4	11.9	11.8	12.7	2.4
		DIY	8	8.2	11.9	11.4	13.2	5.0
		75E	8	10.9	12.4	12.7	15.1	4.1
	HCl demineralisation	EE	8	12.4	14.3	14.0	14.9	2.6
		DIY	8	12.7	13.3	13.7	15.6	2.9
		75E	8	13.3	14.6	15.1	18.1	4.8
	HCl demineralisation +gelatinisation	EE	8	11.5	12.3	12.4	13.9	2.4
		DIY	8*	12.2	16.5	16.7	21.4	9.1
		75E	8	13.7	14.4	14.4	15.4	1.7
Elephant	EDTA demineralisation	EE	8	19.9	24.3	24.2	27.9	8.1
		DIY	8	22.2	24.5	24.5	26.4	4.2
		75E	8	18.8	24.0	23.6	29.1	10.2
	HCl demineralisation	EE	8	23.7	25.6	25.5	27.8	4.1
		DIY	8	23.9	26.2	26.1	27.8	3.9
		75E	8	25.0	29.6	29.2	35.3	10.3
	HCl demineralisation +gelatinisation	EE	8	13.0	20.8	19.9	21.5	8.5
		DIY	8	/	/	/	/	/
		75E	8	22.3	23.5	23.7	25.8	3.4
Mammoth	EDTA demineralisation	EE	8	2.7	3.9	4.2	6.2	3.5
		DIY	8	2.7	3.8	3.7	4.3	1.6
		75E	8	1.7	3.4	3.4	4.5	2.8
	HCl demineralisation	EE	8	3.9	4.8	4.9	6.2	2.3

		DIY	8	5.1	5.6	5.8	7.0	1.8
		75E	8	6.0	7.0	6.9	7.6	1.6
	HCl demineralisation +gelatinisation	EE	8	3.8	4.9	4.7	5.6	1.7
		DIY	8	5.2	6.1	6.2	7.2	2.1
		75E	8	6.7	7.3	7.9	12.0	5.3
Reindeer	EDTA demineralisation	EE	8	9.1	10.3	10.1	10.9	1.7
		DIY	8	8.6	10.0	10.0	10.9	2.2
		75E	8	9.2	9.6	9.7	10.4	1.3
	HCl demineralisation	EE	8	9.0	10.6	11.0	12.8	3.7
		DIY	8	9.1	11.9	11.7	12.8	3.7
		75E	8	12.3	13.2	13.3	14.4	2.1
	HCl demineralisation +gelatinisation	EE	8	9.1	9.8	9.7	10.2	1.1
		DIY	8	10.4	11.2	11.2	12.2	1.7
		75E	8	11.9	12.5	12.5	13.4	1.5

Table 3. Percentage O exchangeability in collagen (X_o) due to exposure to atmospheric moisture. * indicates compromised samples.

	Exposure/drying method	High temperature (120°C)/ Oven (40°C)	Ambient temperature (20°C)/Oven (40°C)	Ambient temperature (20°C)/Freeze drier (c.-60°C)
	Extraction method	X_o	X_o	X_o
Cattle	EDTA demineralisation	3	1	4
	HCl demineralisation	3	0	2
	HCl demineralisation +gelatinisation	3	2	2
	*HCl demineralisation +gelatinisation	32*	21*	20*
Elephant	EDTA demineralisation	5	5	4
	HCl demineralisation	6	7	7
	HCl demineralisation +gelatinisation	3	2	7
Mammoth	EDTA demineralisation	3	1	2
	HCl demineralisation	2	1	2
	HCl demineralisation +gelatinisation	3	2	3
Reindeer	EDTA demineralisation	2	0	2
	HCl demineralisation	2	2	2
	HCl demineralisation +gelatinisation	2	1	2

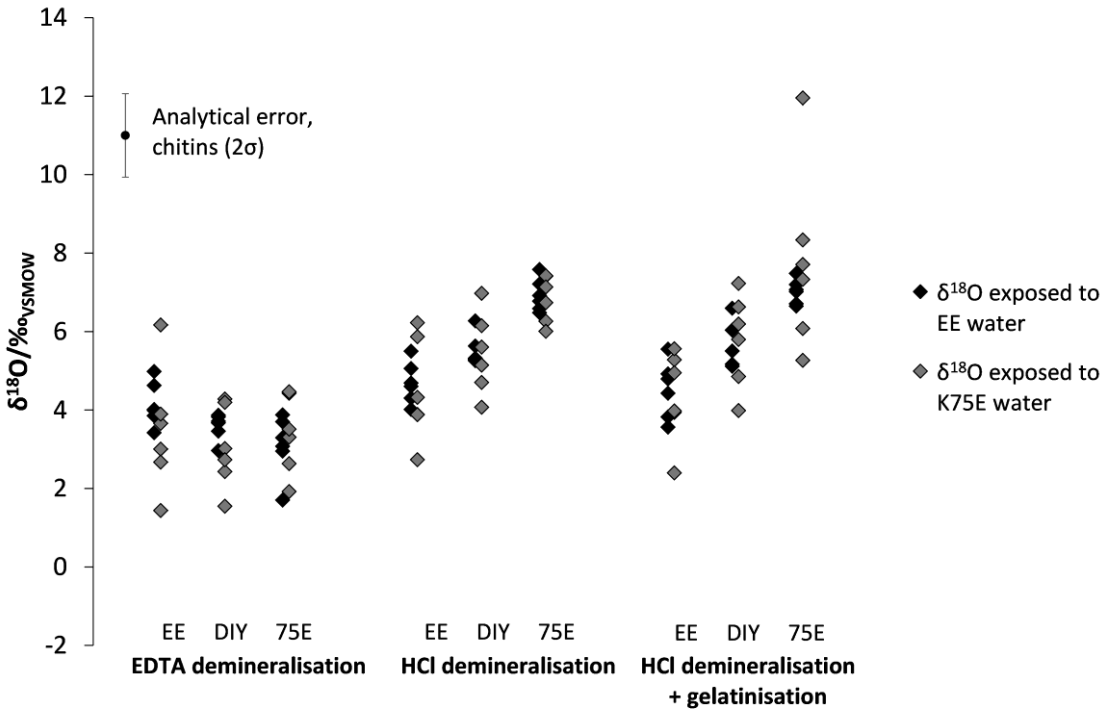

Figure 1. Measured $\delta^{18}O$ values after exposure to atmospheric water for mammoth collagen. Samples are grouped by reagent water (EE, DIY, 75E) within extraction method.

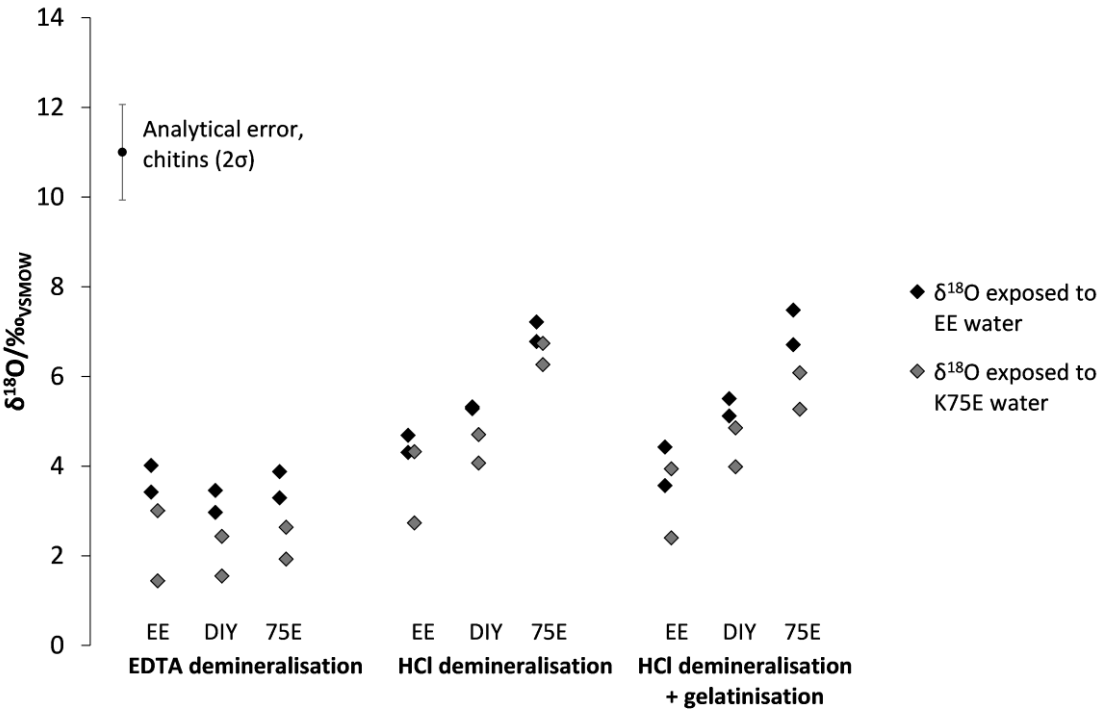

Figure 2. Measured $\delta^{18}O$ values for mammoth bone collagen using high-temperature equilibration.

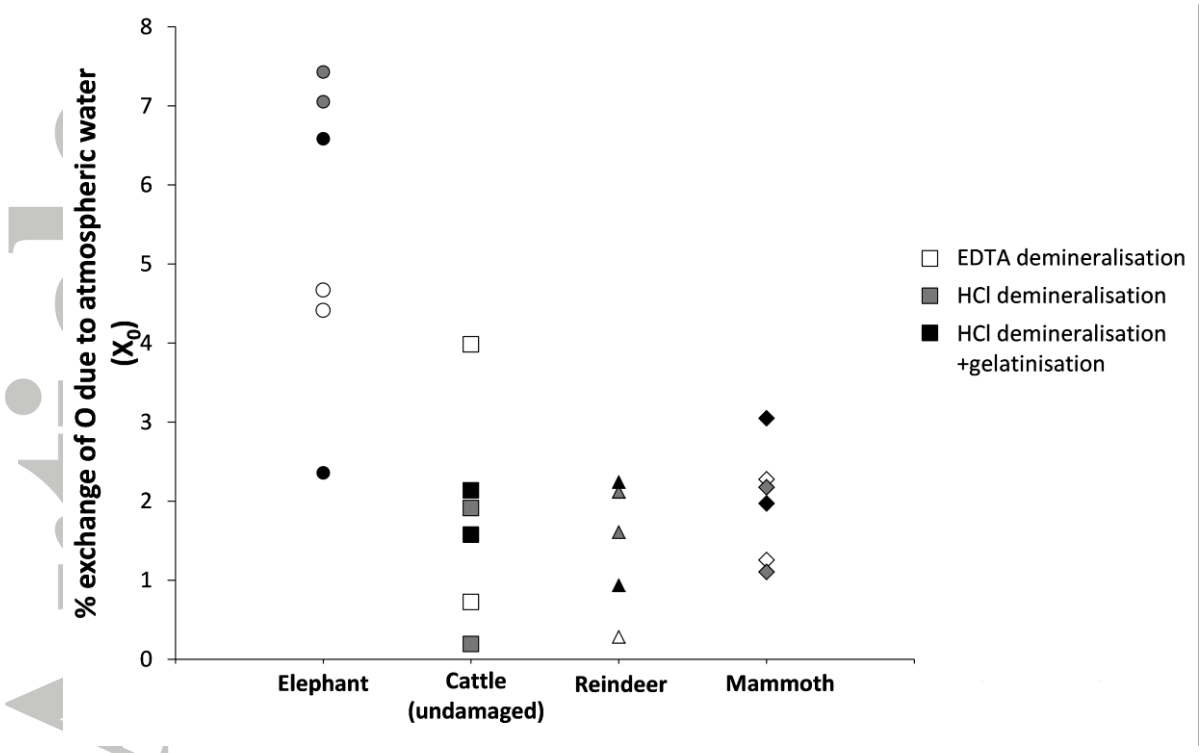

Figure 3. % of O exchange in collagen due to atmospheric water. Compromised cattle samples, and samples equilibrated at high temperature, are excluded.

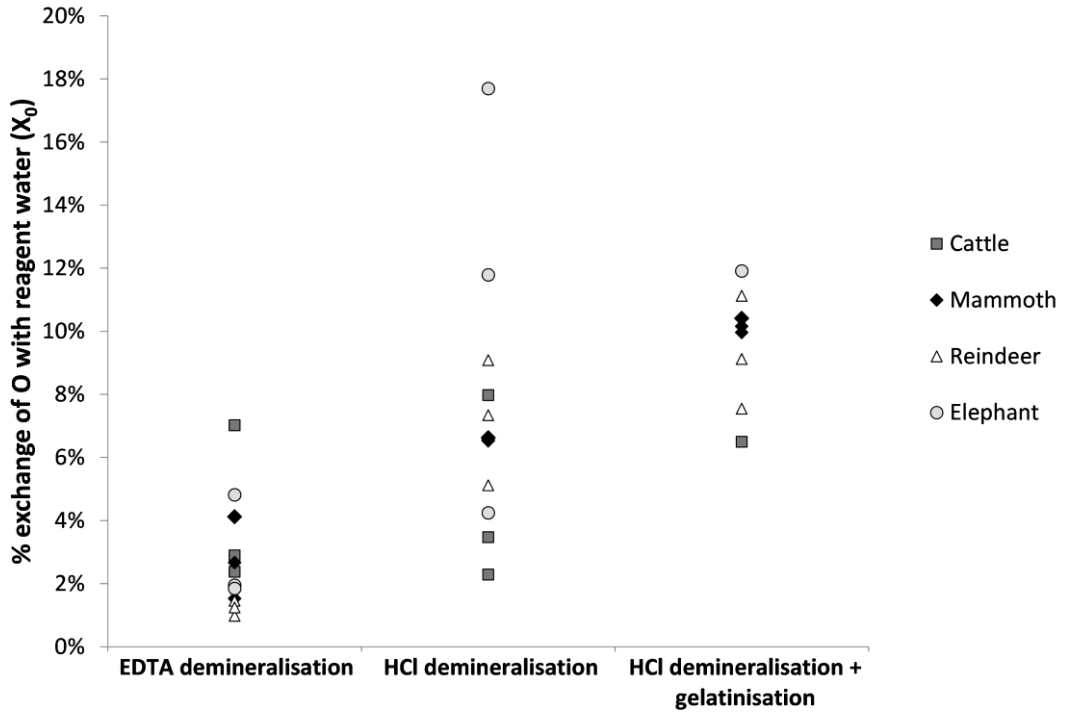

Figure 4. % O exchange in collagen due to reagent water. Compromised cattle samples, and samples equilibrated at high temperature, are excluded.

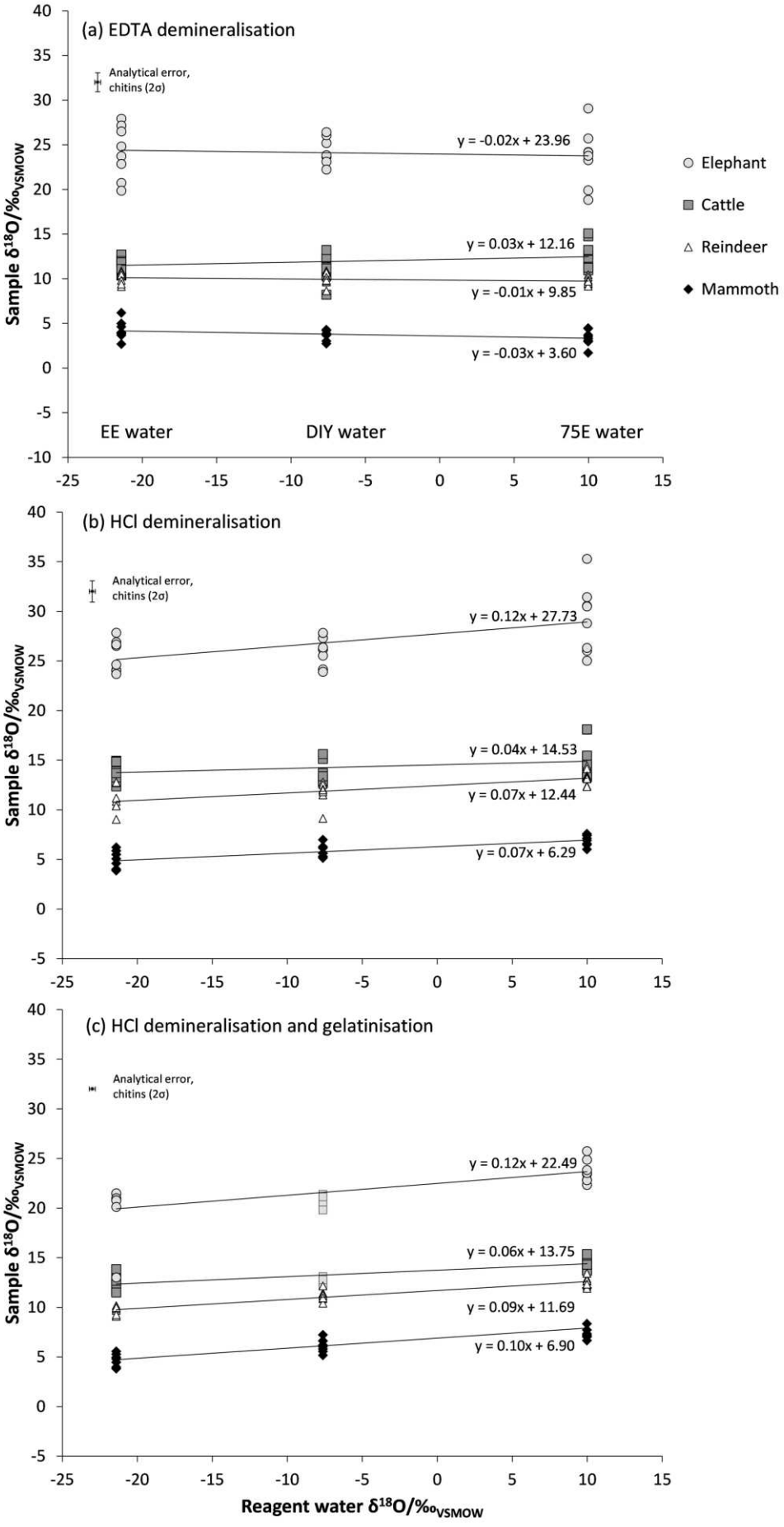

Figure 5. Measured $\delta^{18}O$ ranges for each sample after collagen extraction and atmospheric equilibration, plotted against reagent water $\delta^{18}O$. High-temperature equilibrated samples were excluded. (a) EDTA demineralisation; (b) HCl demineralisation; (c) HCl demineralisation and gelatinisation. Compromised cattle samples are shown in paler grey in figure (c). Linear trendlines are indicative of overall average exchange due to reagents, where the gradient indicates total average X_o.